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Faculdade de Ciências

Departamento de Biologia Vegetal

Impact of seawater acidification in starfish regeneration – a proteomic approach

Mestrado em Biologia Molecular e Genética

Vera Cristina J. Marques

Thesis Dissertation supervised by

Dr. Manuel Pedro Fevereiro

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ACKNOWLEDGMENTS

Em primeiro lugar não poderia deixar de agradecer à Doutora Ana Varela Coelho pela oportunidade de realizar esta tese sob a sua orientação, e por toda a ajuda no desenvolvimento e escrita desta dissertação. Ao Professor Doutor Manuel Pedro Fevereiro, agradeço-lhe por ter aceitado ser meu orientador interno, e pela disponibilidade demonstrada quando precisei.

Às pessoas que me ajudaram a ultrapassar um ano que se adivinhava difícil: Rita Daniela, obrigado pela passagem de conhecimentos laboratoriais e matemáticos (ihih), e por toda a paciência com uma mente nada matemática; Jô, obrigado também pelo que me ensinaste quando nem era suposto, pelas boleias e pelos serviços gratuitos de personal trainer ahah. Às duas, tenho que agradecer as brincadeiras, parvidades, jantares, e dias bem passados, assim como a amizade e os conselhos nada dignos de 'Maria Helena' :p

Catarina, obrigado pelas amostras das tuas estrelinhas, pela disponibilidade demonstrada, e por este apoio final ;)

Obrigado a todos os meus amigos, em especial Manel, Lúcia, Helena, Andreia, Moss, Liliana, Natália, Carina, que de alguma forma contribuíram para que houvesse força para continuar.

E como os últimos são sempre os primeiros, tenho que agradecer ao meu Manel e à minha Maria pela oportunidade de concluir este mestrado, pelo apoio, força e alegria que me dão. À minha carraça Marta Sofia, obrigado por seres a minha melhor amiga e eu ser a tua .. "isto é amor de irmãs"

ABSTRACT

Since the start of the industrial revolution (XVIII century), atmospheric levels of carbon dioxide (CO_2) have been rising at a far greater rate than previously experienced, increasing CO_2 dissolved in seawater. This process is recognized as ocean acidification and can severely damage calcifying organisms. Since echinoderms have an endoskeleton composed of magnesium calcite, they are predicted to be a sensitive *taxa* affected by this acidic environment. Taking into account that some species of this *filo* have a faster regeneration capacity under low pH, analysis of the proteome represents a powerful tool to examine these physiological trait at a molecular level. Trying to strengthen this work on ocean acidification effects, we studied *Asterias rubens'* behaviour by observing their preference in choosing a leading arm when submitted to different regeneration time-points (non-regeneration and regeneration after 1, 4, 9 and 14 days) and at two pH environments (control (CpH) – 8.1 and low (LpH) – 7.7). It was concluded that they do not have a preference leading arm before ablation in both pH environments. Although, one day after ablation they drastically decrease the use of the wounded arms, as expected, while the non-amputated arms did not suffer any changes. Furthermore, after nine days subjected to these conditions, it seems that sea stars recovered completely the movement of the amputated arms. Asterosaponins search was also a vital part of this work, since they are known for having important functions in sea stars. We observed that two of the nine asterosaponins detected in the control pH were also detected in low pH samples, and that their concentration decreased, suggesting that acidic pH may alter their biosynthesis. Proteins from both cell-free coelomic fluid (CFF) and radial nerve cord (RNC) were extracted, digested and further analyzed by nano LC-MS/MS. We were able to identify 298 proteins, being 81% of all proteins identified differentially expressed. A surprising finding was the almost absence of stress proteins in an acidic pH environment.

In this work, we propose a correlation between an acidic environment and asterosaponins' biological effects. The decline in diversity and amount of these biomolecules can be caused by already known decreases in respiratory rate and cholesterol absorption. Although, asterosaponins might have positive effects in predation and reproduction. The detected increase in vitellogenin expression seems to be associated with the amplified spawning event induced by the low levels of asterosaponins at acidic pH, thereby also enhancing reproduction.

Keywords: ocean acidification, *A. rubens*, asterosaponins, behaviour, proteome

RESUMO

Desde o início da revolução industrial (século 18) que os níveis atmosféricos de dióxido de carbono (CO_2) têm aumentado a um ritmo nunca antes registado (de 280ppm para 380ppm, e espera-se que duplique no final do século), essencialmente devido à emissão de gases com efeito de estufa resultantes de atividades humanas. Como consequência, a concentração de CO_2 dissolvido nos oceanos também aumenta, e por sua vez, também a concentração de iões hidrogénio e iões bicarbonato, conduzindo a uma diminuição de pH e de iões carbonato. Este conjunto de reações é designado por acidificação dos oceanos, e pode danificar seriamente o funcionamento de organismos marinhos que de alguma forma utilizam o processo de biocalcificação na sua estrutura corporal. Os equinodermes possuem um endosqueleto composto por calcite de magnésio, têm uma extraordinária capacidade de regeneração, mas também são osmoconformadores. Isto significa que o fluido celómico (CF) que preenche a cavidade corporal é muito semelhante, a nível de composição iónica, ao ambiente externo a que está submetido. Embora também muito idêntico, o pH do fluido celómico é geralmente 0.11-0.58 unidades mais baixo que o pH do exterior, devido a taxas de difusão de CO_2 mais ou menos lentas, que podem aumentar ou reduzir a concentração de dióxido de carbono no CF, respetivamente. Por estas razões prevê-se que os equinodermes sejam um t*axa* sensível a ambientes ácidos. Apesar de se esperar que todas as espécies sejam afetadas de forma negativa, é do conhecimento científico que algumas espécies deste *filo* crescem e desenvolvem-se mais depressa quando expostas a pH ácido, enquanto outras apresentam diminuição de metabolismo ou redução da expressão proteica global. Considerando a diversidade incongruente de resultados publicados, e tendo a estrela-do-mar comum *A. rubens* como espécie-alvo, realizámos análises de expressão proteica para estudar estas observações fisiológicas de uma perspetiva molecular.

Por forma a ter um estudo mais completo sobre o efeito da acidificação nesta espécie, observámos estrelas-do-mar sob diferentes tempos de regeneração (não-amputação e amputação após 1 dia, 4, 9 e 14) submetidas a dois pHs (controlo (CpH) – 8.1 e ácido (LpH) – 7.7), com o propósito de determinar com que braço preferencialmente elas iniciavam a deslocação. Os braços escolhidos para amputação foram os mais próximos à madreporita. As estrelas-do-mar não apresentaram preferência no braço 'líder' antes de amputadas e sob CpH, o que não é consistente com resultados já publicados. No entanto, como esperado, os braços amputados apresentaram uma diminuição do número de movimentos um dia após a regeneração ser imposta, enquanto o número de movimentos dos braços não-amputados permaneceu igual. Após nove dias os movimentos dos braços amputados foram restabelecidos para os valores iniciais (sem amputação), e por isso considerámos que as estrelas recuperaram o movimento normal dos braços. É vantajoso para a

estrela-do-mar que este movimento seja restabelecido o mais rapidamente possível, para que possa, entre outros, alimentar-se e/ou fugir de possíveis predadores.

Para estudar os fatores moleculares que possam estar envolvidos em resposta a uma futura diminuição do pH dos oceanos, foram recolhidos dois tipos de amostra: fluido celómico (dois pHs e 15 dias pós-amputação), e nervo radial (RNC; dois pHs e não-amputação; dois pHs e 15 dias pós-amputação). O CF foi centrifugado para que fossem retirados os coelomócitos, designando-se desta forma fluido celómico livre de células (CFF). Este fluido foi submetido a centrifugação e ultrafiltração para separar a fração de alta massa molecular (proteínas) da fração de baixa massa molecular. Esta última contém asterosaponinas, moléculas que são conhecidas por terem funções importantes a nível de sinalização química mas também a nível digestivo e reprodutivo, e que por isso considerámos essenciais neste estudo. Esta fração foi então sujeita a extração de fase sólida (SPE) com diferentes eluições de acetonitrilo e ácido fórmico para isolamento das asterosaponinas, e posteriormente analisadas por ESI-MS. Os dados foram adquiridos por um espectrofotómetro linear ion trap mass Thermo Finnigan LTQ, controlado pelo *software* Xcalibur v 2.0, também usado na análise dos espectros resultantes da leitura das amostras. Todas as asterosaponinas investigadas estavam presentes no pH controlo, mas apenas duas delas foram detetadas em estrelas-do-mar submetidas a pH ácido. Para além disto, as suas concentrações diminuíram em pH ácido. Tendo em conta que a *A. rubens* apresenta um baixa taxa de respiração após 27 dias exposta a pH 7.7 do que quando submetida a pH 8.1, podemos assumir que este decréscimo irá alterar a disponibilidade de ATP, e consequentemente comprometer a biossíntese de asterosaponinas. Por sua vez, a diminuição destas biomoléculas poderá ter efeitos negativos a nível digestivo, através da redução da absorção de colesterol, mas poderá também ter efeitos positivos ao nível da predação.

As proteínas do CFF foram extraídas e precipitadas com recurso a ácido tricloroacético e beta-mercaptoetanol, enquanto as proteínas do RNC foram extraídas e precipitadas recorrendo a azoto líquido e a um buffer suplementado com SDS, Tris-HCl, DTT, um cocktail anti-protease e PMSF. A quantificação das proteínas do CFF e RNC foi feita com recurso ao QuantiPro BCA Assay KIT 0.5-30µg/mL protein, e a digestão, embora diferente, teve como base o uso de redutores de pontes persulfureto (DTT e TCEP), iodoacetamida para alquilação das cisteínas e tripsina para quebrar a proteína em péptidos. As amostras digeridas de ambos os tecidos foram analisadas por nano LC-MS/MS. Foram identificadas 213 proteínas no RNC, mas apenas uma estava presente em todas as condições estudadas. Por esta razão, podemos concluir que o procedimento experimental deve ser otimizado. A análise do CFF permitiu identificar 298 proteínas, das quais 19% eram comuns às duas condições de pH. De acordo com estes resultados, 81% de todas as proteínas identificadas estavam diferencialmente expressas. Comuns a ambas as condições de pH, e como esperado, foram detetadas actinas, tubulinas e ubiquitinas. A quantificação realizada permitiu observar um aumento

de expressão significativo apenas em 2 proteínas no pH ácido: alfa-tubulina e vitellogenina 1. A primeira está envolvida na formação do citoesqueleto, e por isso, será importante numa fase de rápida proliferação celular. A segunda proteína é responsável pelo transporte de lípidos para os oócitos, e o seu aumento de expressão parece aumentar o número de eventos de desova em estrelas-do-mar. Os baixos níveis de asterosaponinas presentes em pH ácido também parecem favorecer este acontecimento, aumentando desta forma, o sucesso reprodutivo.

Com base em estudos recentes realizados em corais e em zooplâncton, previa-se um aumento na expressão de proteínas de stress em condições de pH ácido. No entanto, no presente estudo, as estrelas-do-mar submetidas a esta condição de pH, exibiram apenas uma proteína de stress. Para melhor compreensão dos efeitos da acidificação dos oceanos em cada uma das proteínas aqui identificas, será necessário realizar estudos mais detalhados para avaliar o impacto biológico da expressão proteica diferencial que observámos.

Palavras-chave: acidificação, *A. Rubens*, comportamento, asterosaponinas, proteoma

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INDEX OF ABREVIATIONS

CO_2	Carbon dioxide
GHG	Greenhouse Gas
H_2CO_3	Carbonic acid
HCO_3^-	Bicarbonate ion
CO_3^{2-}	Carbonate ion
OA	Ocean acidification
$CaCO_3$	Calcium carbonate
CF	Coelomic Fluid
RNC	Radial Nerve Cord
CFF	Cell-free coelomic fluid
MS	Mass spectrometry
HPLC-MS	High Performance Liquid Chromatography linked with MS
ESI	Electrospray Ionization
LC-MS	Liquid chromatography linked with MS
MS-MS	Tandem-Mass spectrometry
SPE	Solid phase extraction
FA	Formic acid
ACN	Acetonitrile
TCA	Trichloroacetic acid
BA	Ammonium bicarbonate
CID	Collision induced dissociation
MWCO	Molecular weight cut-off
DTT	Ditiotreitol
NAC	N-Acetyl-L-cysteine
TCEP	Tris (2-carboxyethyl) phosphine
CpH	Control pH
LpH	Low pH
PA	Post-amputation/ablation
NA	Non-ablation
UF	Ultrafiltration
m/z	Mass to charge ratio
GO	Gene ontology
BP	Biological process
MF	Molecular function
HSP	Heat shock protein

INTRODUCTION

- **Ocean Acidification**

Since the start of the industrial revolution (XVIII century), atmospheric levels of carbon dioxide (CO_2) have been rising at a far greater rate (from 280 to 380ppm, and is expected to double by 2100) than previously experienced in Earth's history, primarily as a result of Greenhouse Gas (GHG) emissions from human activities [1] (**Fig. 1, 2**). Consequently, CO_2 dissolved in seawater also increase, and will combine with water molecules to form carbonic acid (H_2CO_3). This acid dissociates, and release a hydrogen ion (H^+) and a bicarbonate ion (HCO_3^-) until the chemical equilibrium is reached. Also, HCO_3^- releases H^+ and a carbonate ion (CO_3^{2-}). Most of the H^+ released by the carbonic acid will subsequently combine with carbonate ions to form additional bicarbonate ions, thereby reducing the pool of carbonate ions [2]. This decrease has significant consequences for the chemistry of carbonate minerals that generally constitute shells, plates or skeletons of marine biota. The formation and dissolution of carbonate minerals can be represented as [1]:

Mineral formation

$$CaCO_3 \leftrightarrow [Ca^{2+}] + [CO_3^{2-}]$$

Dissolution

Once formed, calcium carbonate ($CaCO_3$) in these structures will dissolve back into the water unless the surrounding seawater contains sufficiently high concentrations of carbonate ions. In summary, the chemical changes in seawater resulting from increased atmospheric CO_2 concentrations include increases in the concentrations of dissolved (or aqueous) carbon dioxide, hydrogen ions, and bicarbonate ions, and decreases in the carbonate ion concentration and pH [3] (**Fig. 3**)**.** This group of related processes is denominated **ocean acidification (OA)** [4]. Some areas are

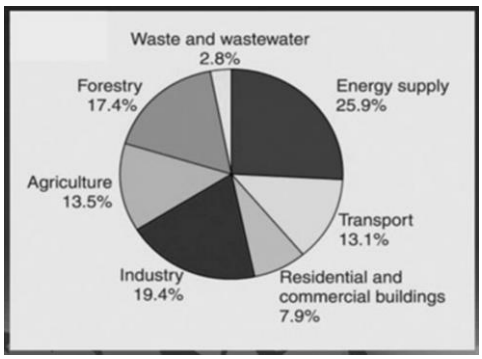

Figure 1. Contribution to Earth's climate change of different anthropogenic sectors (sources of greenhouse gas emissions) [56].

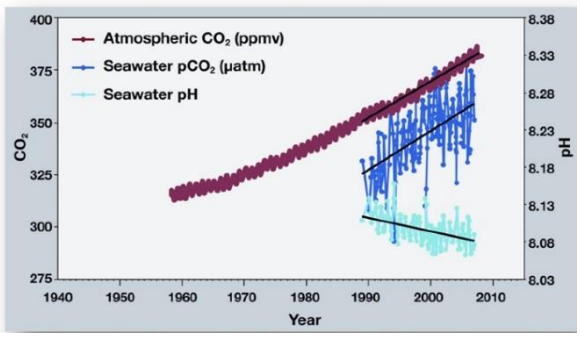

Figure 2. Correlation between rising levels of CO_2 in the atmosphere at Manua Loa, Hawai with rising CO_2 levels in the nearby ocean at Station Aloha. As more CO_2 – and consequently H^+accumulates in the ocean, the lower will be the pH [55].

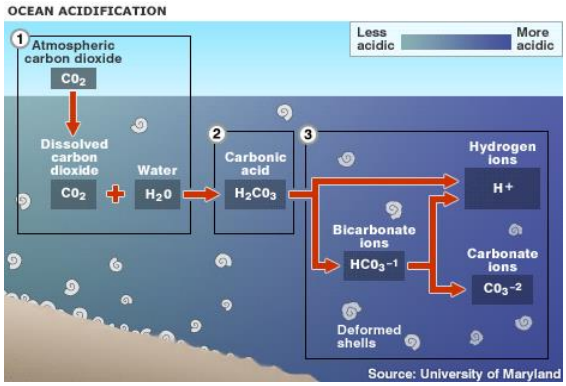

Figure 3. Chemical reaction responsible for the ocean acidification [57].

regularly subjected to this process, such as upwelling regions, hydrothermal vents or intertidal zones [5] where organisms like echinoderms live in. Assuming that is difficult to achieve mineral formation under an acidic pH, calcifying echinoderms are predicted to be a highly sensitive *taxa* to ocean acidification [6]. Dupont et al (2010) [7] under the assumption of a seawater pH predicted to occur by the year 2100 (ΔpH≈-0.4 units) tested two hypotheses: (1) owing to their calcite skeleton, sea star C*rossaster* papposus would be negatively affected or (2) that owing to their lecithotrophic larvae, they may be more resistant to environmental changes. The larvae and juveniles raised at low pH grew and developed faster, with no negative effect on survival or skeletogenesis within the time frame of the experiment (38 days). These results allowed the authors to reject the first hypothesis. Furthermore, this species seem to be not only more resistant to OA, but is also performing better. Though, it is important to remember that negative effects may appear in the longer term or on parameters not measured in this study (e.g. calcification rate). Also, the brittle star *Amphiura filiformi* can increase the rates of metabolism and the ability to calcify to compensate for increased seawater acidity [8]. Although, there are also studies where negative effects are reported (collard 2013).

- **Echinoderms**

The ability to replace lost body parts or form a new functional tissue varies widely among animals, and this variability of regenerative potential has long perplexed biologists [9]. Since the earliest experimental studies on regeneration during the 1700s, it has been clear that animals, like amphibians, hydras, planarias, echinoderms or even humans differ substantially in their regeneration ability [10], in part because it can occur at multiple levels of biological organization (cell, tissue, internal organs, structure, or the whole body). Furthermore, it can be triggered by a variety of causes, occur at different stages of the life cycle, proceed via a diversity of developmental processes and produce structures of variable fidelity relative to the original [9]. Among others, echinoderms (Phylum Echinodermata) - which belong to the superphylum Deuterostomia like vertebrates - is one of the groups usually chosen to study regeneration. It is divided in five classes: Echinoidea (sea urchins), Asteroidea (sea stars), Ophiuroidea (brittle stars), Holothuroidea (sea cucumbers) and Crinoidea (feather stars) [11] (**Fig. 4)**. They are exclusively marine and widely distributed in all oceans, from the intertidal to the abyssal regions.

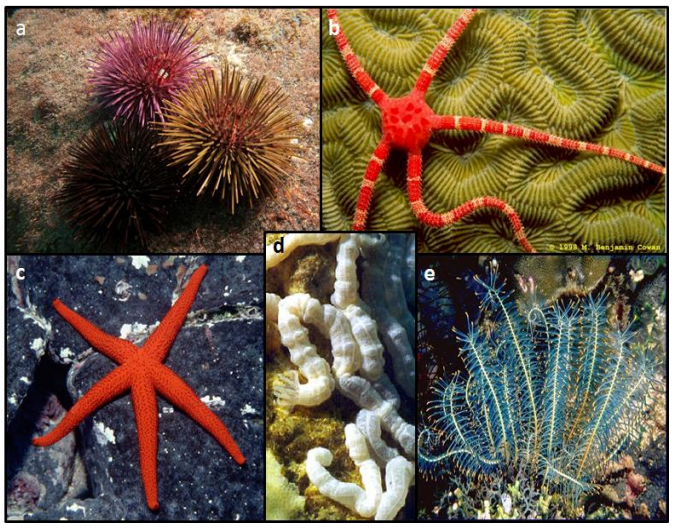

Figure 4. Examples of Echinoderms. a) Echinoidea, b) Ophiuroidea, c) Asteroidea, d) Holothuroidea and e) Crinoidea [58], [59]

Echinoderms can be carnivorous, detritus foragers, or planktonic feeders, such as the sea star, some sea cucumbers and basket stars (ophiuroids), respectively. All classes present radial symmetry – although juvenile states present bilateral symmetry – usually with five or more arms, and each of these segments contain an exact replica of all internal organs [12]. They lack morphologically differentiated excretory organs and have a poor ability to osmo and ion regulate (osmoconformers) and that is why their **coelomic fluid (CF) -** the main circulatory medium - has an ionic composition similar to that of seawater [13]. Locomotion is accomplished using tentacle-like structures called tube feet, which are hydraulically controlled by a vascular system that moves water through canals of their small muscular tubes [14]. Along with this water vascular system, the CF ensure gas transportation and is involved in cell-free (humoral) immune responses due to the presence of proteins secreted by the coelomocytes or by the surrounding tissue like the **radial nerve cord (RNC)** [13]. This part of the nervous system is located at the oral surface of animal, where it is exposed to the outside environment. The RNC of each arm join together forming the circumoral nerve ring, which surround the mouth [15]. The fraction free of coelomocytes (acquired by centrifugation) was designated by us **cell-free coelomic fluid (CFF)** and is extremely rich in secreted molecules, like growth factors, hormones and neuropeptides, which are involved in cell signaling processes [14].

Echinoderms usually have separate sexes with no evident sexual dimorphism, and reproduction is typically achieved by external fertilization, with eggs and sperm freely released into seawater. The resulting embryos and larvae live as part of the plankton, or for others species, the eggs may be stuck to the undersides of rocks. Some species of sea star are able to reproduce asexually as adults either by fission of their central discs [16] or by autotomy (self-amputation behaviour) of one or more of their arms. One of most important features of Echinoderms is the presence of an endoskeleton in

post-metamorphic stages, which in most species occupies a significant part of their dermis. This endoskeleton is composed of magnesium calcite (Ca-Mg-CO_3) by a process called biocalcification that is biologically mediated and involves ion transport pathways in echinoderms. Although Echinodermata are osmoconformers, their coelomic fluid's pH is usually 0.5-1.5 units lower because of a high CO_2 content, due to the slow elimination of this gas which is dependent on diffusion gradients for exchange with seawater. However, Collard et al., (2013) showed that the CF of the common sea star *A. rubens* was in equilibrium with seawater's pH after twenty four hours exposure to pH 7.7, with a slight difference of less 0.21-0.40 units [17]. Nevertheless, this *filo* present significant morphological and physiological plasticity that allows them to live in different environments, presenting different growth rates, reproductive periodicity and environmental tolerances [11].

- *Asterias rubens*

The common sea star (Linnaeus, 1758; **figure 5, table 1**) is the most prevalent sea star on the north-east Atlantic, but can also be found from Arctic Norway, along Atlantic coasts to Senegal, and only found occasionally in the Mediterranean [18]. It is found on a variety of hard substrata and occurs from the upper tide mark down to about 400m, rarely down to 650m. It can also be found on mussel beds offshore.

Figure 5. *Asterias rubens* [18]

They are usually orange, but may vary between pale brown to violet and exceptionally can grow up to 52cm in diameter [19]. Spreading from a central disc with a ventral mouth and a dorsal anus it has 5 pointed arms. The upper surface is covered in spines which are surrounded by pedicellariae, tiny pincers that discourage predators and keep detritus and other particulate matter from settling on the animal. The most apical tube feet of each arm are modified light sensory organs and the rest are used for locomotion and capture prey, which consists of mainly bivalves like mussels and oysters but also roes, carrion, gastropods, crabs, polychaete worms and other echinoderms like sea urchins, starfishes, brittle stars and sea cucumbers [20].

From February to April the females release, through ventral pores, about 2.5 million eggs each and stimulate the males chemically to release their sperm, therefore fertilization is external. *A. rubens* reaches maturity with one year but can live up to 5-10 years [20].

Table 1. *A. Rubens* scientific classification.

Kingdom	**Animalia**
Phylum	Echinodermata
Class	Asteroidea
Order	Forcipulata
Family	Asteridae
Genus	Asterias
Species	rubens

- **Asterosaponins**

Mainly produced by plants, but also found in lower marine animals like sea stars and some bacteria, saponins are naturally occurring surface-active glycosides [21]. Their name is derived from their ability to form stable, soap-like foams in aqueous solutions. These molecules consists of a sugar moiety usually containing glucose, galactose, glucuronic acid, xylose, rhamnose or methylpentose, glycosidically linked to a hydrophobic aglycone (sapogenin) which may be triterpenoid in nature. They are of high interest due to their hemolytic, cytotoxic, anti-bacterial, anti-fungal, anti-viral and anti-tumor properties [21].

Three categories of saponins have been identified in sea stars, polyhydroxysteroids glycosides, macrocyclic saponins and asterosaponins [22, 23], but only the latter was found in *A. rubens* [24]. Asterosaponins are $\Delta^{9(11)}$-3β, 6α-dioxygenated steroids with a sulfate group attached at C-3 and an oligosaccharide chain containing five or six sugar units at C-6, and can be characterized by a wide degree of structural variability because individual compounds belonging to this class differ in their steroidal side chain and/or in their sugar moiety. Yasumoto and his group (1966) surveyed all five classes of echinoderms and found that only the holothurians and the asteroids elaborate these compounds – responsible for their toxicity –, while the echinoids, ophiuroids, and crinoids do not. This finding reinforced the suspected close phylogenetic relationship of sea cucumbers and sea stars even though holothurians have triterpenoid aglycones while asteroids have steroid aglycones [25]. Asterosaponins are also known for being responsible for causing an 'escape response' on many organisms when in the presence of, or contact with sea stars, thereby reducing predation success [26]. Specifically in asteroids, it is generally considered that saponins may have a role also in digestion, reproduction and chemical signaling [21].

- **The proteomic approach**

Recent studies suggest an uncoupling between transcript and protein levels in coccolithophores under ocean acidification [27] that are in agreement with older studies performed in mammalian, yeast and bacterial cells [28; 30]. The coupling of these levels can be as low as 40%, depending on the system [31], therefore knowledge of protein expression pattern is necessary to understand the direct link between ocean acidification stress and organism's response. The proteome, which is the expressed protein complement of the genome, varies among tissues and over time, but represents the final and stable product of many redundant gene expression processes [32; 33]. For example, to address this question Dineshram et al. (2012) [34] performed 2-DE coupled with mass spectrophotometry to compare the global protein expression pattern of the wild Pacific oyster *Crassostrea gigas* larvae exposed to normal and high-CO_2 conditions. Exposure to OA during 4 days

after fertilization resulted in marked reduction of global protein expression with a decrease or loss of 71 proteins (18% of the expressed proteins in control) indicating a wide-spread depression of metabolic gene expression. Importantly, expression of proteins related to calcification and cytoskeleton production appears to be severally suppressed by OA.

- **High Performance Liquid Chromatography linked with MS (HPLC-MS)**[35]

As in many cases, our compounds of interest are found as a part of a complex mixture, and the role of the chromatography technique is to provide separation of the components of that mixture to allow their identification and/or quantitative determination. Quantification is achieved by comparison of the intensity ('peak' area) of response from samples of different experimental conditions.

The separation of the components are associated with two phases: mobile and stationary. The former is a liquid (in which the analytes are soluble) delivered under high pressure to ensure a constant flow rate and thus reproducible chromatography, while the latter is packed into a column capable of withstanding the high pressures which are necessary. The majority of HPLC separations hyphenated with MS utilize reversed-phase chromatography, i.e. the mobile phase is more polar then the stationary phase, therefore analytes more polar elute more rapidly than the less polar ones. HPLC system is composed by a pump that delivers the mobile phase at a constant flow rate, an injector, a chromatography column and a detector.

- **Electrospray Ionization (ESI)**

The majority of ionization techniques employed in the analysis of biomolecules are 'soft ionization's techniques which provide primarily molecular ions that occur at relatively high values of mass-to-charge ratio (*m/z*), rather than fragment ions which occur at relatively low *m/z* values. There are other ionization methods but we will only refer to the one used. ESI technique consists of a liquid with the analyte of interest, which enters the ES chamber through a stainless steel hypodermic needle [36] and pass through a capillary, at atmospheric pressure, maintained at high voltage. The liquid is dispersed and forms charged droplets which are desolvated, usually by a stream of nitrogen, as they pass through the atmospheric-pressure region of the source towards a counter electrode. At this point, the droplet deforms as the electrostatic repulsion of similar charges, in an ever-decreasing droplet size, becomes more powerful than the surface tension holding the droplet together. The droplets undergo 'Coulombic explosion' producing more stable droplets with a radius of approximately 10% of that of the parent droplet. These enters directly to a heated capillary and then are transferred through a series of focusing lenses into the mass spectrometer.

This system can allow quantification, but to obtain the best precision and accuracy during quantitative measurements an internal standard should be used. Analytical signals from both the analyte and the internal standard are measured and the ratio of these two signal intensities ('peak' area) are used to generate the calibration graph and to determine the amount of analyte present.

- **Tandem-Mass spectrometry (MS-MS)**

This way of generate structural information is applicable to all forms of ionization and covers a number of techniques in which one stage of mass spectrometry is used to isolate an ion of interest and a second stage is then used to probe the relationship of this ion with others from which it may have been generated or which it may generate on decomposition. There are a large number of different MS-MS experiments that can be carried out, for example, the product-ion scan, the precursor-ion scan, the constant-neutral-loss scan and selected decomposition monitoring.

In this work, the product-ion scan was used to asterosaponins' analysis, where the ion-trap generate product ions from a selected precursor ion, and any of these 'product' ions may be isolated, dissociated and a further product-ion spectrum obtained. In general, the first stage of mass spectrometry is performed to isolate an ion of interest and in the second stage, fragmentation of the ion is effected, usually by collision with gas molecules in a collision cell. A mass spectrum of the product (fragment) ions is then provided. This is termed MS-MS-MS or MS^3. This process can be repeated, being designated by MS^n.

- **Nano LC-MS/MS**

The main difference from LC-MS to Nano-LC is that the latter has managed a gain in sensitivity that allows the analysis of peptide mixtures in sample-limited situations (e.g., proteolytically digested proteins isolated by two-dimensional gel electrophoresis). This nano system allows the use of small amounts of samples due to its lower flow rate. This is possible because of the reduced column diameter comparing to the conventional column diameter in LC-MS.

- **Analysis softwares**
    - *Excalibur 2.0*

Is a complete quantitative and qualitative analysis software package that allows data acquisition specifically for analytes of interest, perform confirmatory library searches, and determine the concentration of analytes in samples.

- *MaxQuant 1.5.2.8*

Is an integrated suite of algorithms specifically developed for high-resolution, label-free quantitative MS data, including protein identification.

- *ProteinScape 3.1 Software*

ProteinScape is a Bruker Daltonics's central bioinformatics platform, developed to provide researchers with sophisticated tools for the analysis and evaluation of proteomic data. This software database efficiently organizes data (for example, LC-data, gel data, mass spectra, process parameters, search results, and data evaluation) for all types of proteomics projects. Essentially, it identifies proteins on the basis of peptide mass spectra. These masses are analyzed, assigned a sequence, and a search in protein databases is made for the presence of the peptide sequences. The higher the scores of the peptide sequences identified in the candidate protein, the higher the confidence in the identification.

- *Blast2GO 3.1.2*

Blast2GO (B2G) is a bioinformatics platform for high-quality functional annotation and analysis of genomic datasets. The software identifies already characterized similar sequences, and transfers its functional labels to the uncharacterized sequences. In this manner, it is possible to obtain functional information for a whole dataset much faster than through experimentation.

AIM OF THE STUDY

Under the paradigm that calcification will be the major biological process impacted by near future OA, it is often hypothesized that echinoderms will be negatively affected. Although, it is already known that some species have a faster regeneration recovery under low pH, and there may be a molecular reason behind it. Therefore, this work had three main purposes to evaluate the effect of ocean acidification using two environmental pH values: 1) study how regeneration affects sea stars arms' use, 2) compare CFF asterosaponins' composition in fifteen days post-amputated sea stars, and finally 3) study proteins expression levels in CFF and RNC using a proteomic differential approach.

- **Animal trials and regeneration induction**

Sea stars were collected at low tide and transported to the Sven Lovén Center of Marine Sciences, Kristineberg (Sweden) on September 2012, and kept in flowing natural deep seawater under natural temperature and light regime (pH 8.06 ± 0.07 units), salinity 32%, and alkalinity of 2.18 ± 0.02mM as measured following Sarazin et al. (1999)[37]. Sea stars were visually inspected and only selected for the experiments if no previous signs of regeneration were present, such as different arm size. They were fed *ad libitum* with frozen mussels. For the experimental manipulation, we selected a seawater pH (ΔpH ~ -0.4 units) predicted to occur by the year 2100 [4] and it was maintained in each aquarium using a computerized control system (AquaMedic) that regulated pH by the addition of pure gaseous CO_2 directly into the experimental tank. The effect of two water pH-levels, pH=8.1 (pCO2 = 372ppm; Ωca = 3.2; Ωar = 2.0) and pH 7.7 (pCO2 = 930ppm; Ωca = 1.5; Ωar = 1.0), were tested on sea star arm regeneration. The former condition corresponds to control and the latter to ocean acidification conditions. After anesthetizing starfish with 4% (w/v) magnesium chloride in seawater, regeneration was induced by amputation of the arm tip at 2/3 of the way down to the arm, with 2 arm tips amputated per animal (arms adjacent to madreporite, **figure 6**). In total, ten experimental groups were assayed (**Table 2**), including two pH conditions (control pH – CpH; low pH - LpH), two physical conditions (amputated/ablation - A#; non-amputated/non-ablation - NA) and in 4 post-amputation (PA) time points (1, 4, 9 and 14 days - A1, A4, A9 and A14, respectively).

- **Behaviour trials**

The aim of these trials was to access recovery of lost mobility after sea star arm tips ablation. Each animal was assayed individually, after positioned in the center of a tank maintained in the above described seawater conditions. For 2 minutes it was observed with which arm(s) the animal preferentially leaded its displacement. Two arm groups were assigned: amputated arms (AA) and non-amputated arms (NAA).

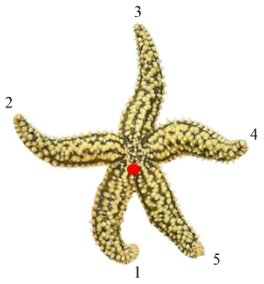

Figure 6. How arms were designated according to madreporite (red circle).

Table 2. Data compilation of tissues analyzed, number of animals used, experimental conditions performed and respective group designation. All these data collection were acquired from the same sea stars.

Sample type	Number of animals used	Experimental group		Experimental Group Designation
CFF	11	15 days post-ablation	Control pH	CpH/A15
	13		Low pH	LpH/A15
RNC	8	15 days post-ablation	Control pH	CpH/A15
	14		Low pH	LpH/A15
	7	No Ablation	Control pH	CpH/NA
	6		Low pH	LpH/NA
Behaviour	60 (30 in each pH condition)	No Ablation	Control pH	CpH/NA
			Low pH	LpH/NA
		24 h post-ablation	Control pH	CpH/A1
			Low pH	LpH/A1
		4 days post-ablation	Control pH	CpH/A4
			Low pH	LpH/A4
		9 days post-ablation	Control pH	CpH/A9
			Low pH	LpH/A9
		14 days post-ablation	Control pH	CpH/A14
			Low pH	LpH/A14

- **Sample Collection**

To collect the RNC, we followed a procedure previously described by Franco et al., (2012) [38]. The internal fluid of the sea star was collected by puncturing the animal epidermis at the arm tip with a needle and collecting the fluid by gravity into an ice cold recipient. After collection and to prevent endogenous proteolysis, both tissues were immediately immersed in an ice cold solution of PBS supplemented with a protease inhibitor cocktail (SIGMAFAST Complete Inhibitor Tablets), flash frozen in liquid N_2 and conserved at −80ºC until further use. Previous to freezing and to avoid coelomocytes lysis, low speed centrifugation (800xg, 10 minutes) was used to separate the coelomic fluid from the circulating cells (CFF fraction). Pools were prepared for both tissues as described in **Table 2**.

- **Extraction of CFF proteins and asterosaponins**

Nine mL of each pool of CFF were subjected to ultrafiltration (UF) using a pre-rinsed centrifugal filtration unit (5kDa MWCO, Vivaspin 20, VWR). Filters were washed with water to remove traces of glycerin and sodium azide. Samples centrifugation was performed at 20ºC and 7000xg. The high

molecular mass fraction (> 5kDa) was stored at -80ºC for posterior differential proteomics. The low molecular mass fraction (<5kDa) was used to perform solid phase extraction (SPE) for separation of asterosaponins. Previous to use, SPE cartridges (Strata-X 33u polymeric reversed phase, Phenomenex) were inserted into a SPE vacuum manifold and washed with 3 mL methanol and 3 mL 5% (v/v) formic acid (FA). Filtrates obtained from ultrafiltration were sequentially eluted from SPE cartridges with 1 mL of increasing acetonitrile (ACN) concentrations (15, 25, 35, 45, 55 of 75% ACN (v/v) containing 5% FA (v/v)). Resultant fractions for each biological replicate were stored overnight at 4ºC, vacuum dried (SpeedVac concentrator, Thermo) and resuspended in 5% (v/v) FA. After use, SPE cartridges were regenerated with 100% (v/v) ACN.

CFF proteins were precipitated overnight at 4ºC with 7.5 mL 10% (w/v) trichloroacetic acid (TCA) and 0.07% (v/v) β-mercaptoethanol. Then, they were centrifuged 10 min at 4ºC and 16 000xg and washed three times with 70% acetone (v/v) containing 0.7% β-mercaptoethanol (v/v) to complete removal of TCA. Next, samples were vacuum dried and resuspended in 50µL of 6M urea 50mM ammonium bicarbonate (BA).

- **RNC proteins extraction**

The FASP protocol (adapted from [39]) was used for RNC protein extraction and tryptic digestion. The tissue (~100mg) was kept frozen in liquid N_2 and placed in a previously chilled Teflon sample chamber containing four stainless steel beads (5mm diameter). The chamber was placed in a Mikro-Dismembrator (Sartorius) and set to 3000rpm for 60s. To avoid sample loss, the resulting powder (still in a deep frozen state) was resuspended in 500µL of hypotonic lysis buffer (5% SDS, 100mM Tris-HCl pH 7.6, 100mM DTT, supplemented with 1X protease inhibitor cocktail and 1mM PMSF). The resulting product was incubated 5 minutes at 95ºC and centrifuged to remove cellular debris and insoluble material (16 000xg, 10 min, 20°C). The supernatant was transferred into a 10kDa MWCO filter unit (Amicon Ultra 0.5mL) and 200µL of Buffer UA (8M urea in 100mM Tris-HCl pH 8.5) was added. A centrifugation was performed at 14 000xg, 20ºC for 15 min, the filtrate discarded and 200µL of Buffer UA added again to the filter unit. This washing step was repeated 10 times. A last centrifugation was made, the filtrate discarded and the remaining sample subjected to quantification.

- **Protein quantification**

Quantification of CFF and RNC protein extracts were performed by QuantiPro BCA Assay KIT 0.5-30µg/mL protein (Sigma-Aldrich).

- **Tryptic digestion of CFF and RNC Proteins**

After extraction and quantification, the proteins extracts of the CFF and RNC were digested for nano LC-MS/MS analysis. The pH of **CFF** samples was adjusted to 8-8.5, added 0.7µL of 700mM DTT and incubated at room temperature for 1h. For alkylation of the cysteines, it was added 2.1 µL of 700mM Iodoacetamide and incubated for 30 minutes at room temperature in the dark. For iodoacetamide quenching, it was added 3.7 µL of 500mM N-Acetyl-L-cysteine (NAC) and the samples were incubated 15 minutes at room temperature. To dilute the urea, 242.95 µL of 50mM BA were added and 2µL of trypsin to digest the proteins. The samples were incubated at 37ºC overnight. Finally, FA enough to have a final concentration of 0.5 % was added and the samples stored at -20ºC.

To achieve **RNC** proteins digestion 100µL of Buffer UA containing 27mM TCEP were added to the filter unit in order to reduce the disulfide bridges. Posteriorly it was mixed at 600rpm for 1 min and incubated 20 min at room temperature. A centrifugation was performed at 14 000xg for 10 min, the filtrate discarded and 100µL of Buffer UA added to the filter unit. The samples were centrifuged again at 14 000xg for 15 min, the filtrate discarded and 100µL of alkylation solution (50mM Iodoacetamide in Buffer UA) added. Mixing 1 min at 600rpm and incubation at room temperature in the dark for 20 minutes was performed. An additional centrifugation (14 000xg, 10 min) was made, the filtrate discarded and 100µL of Buffer UA added. This step was repeated 2 additional times. Another centrifugation was performed, the filtrate discarded and 100µL of 50mM BA added. This step was repeated 2 additional times. One more centrifugation was made and 40µL of 0.05µg/µL tripsin and 0.8µL of 100mM $CaCl_2$ were added to the filter unit that was then transferred to a new collection tube. Samples were vortexed at 600rpm for 1 min and then incubated at 37ºC for 14h. Posteriorly, the samples were centrifuged 15 min at 14 000xg (the filtrate was not discarded because it had the digested peptides) and 100µL of 50mM BA were added. This step was repeated 2 additional times and a last centrifugation was performed. FA was added to the filtrate to have a 5% final concentration and the filter discarded. The peptides were vacuum dried and resuspended in 100µL of 0.2% FA. The samples were stored at -80ºC until further analysis.

- **Analysis of asterosaponins by ESI-tandem MS**

Only the 55% and 75% acetonitrile elutions of the low molecular mass CFF fraction were analyzed due to previous non-published experiments, where not significant intensities for asterosaponins were detected. ESI-MS/MS data were acquired with a Thermo Finnigan LTQ linear ion trap mass spectrometer. Before analysis, 30µL (v/v) of methanol were added in order to facilitate the gas phase ion formation during the electrospray ionization process. Ten µL of 10mg/L peptide (Ae-VFFAED-NH_2) was used as standard for relative quantification. The capillary temperature was set to 275ºC, the spray voltage was set to 5kV for negative mode. The tune parameters optimized (*m/z*

766) were set to -43V and -113.89V for capillary and tube lens voltage, respectively. The sample solutions were infused at a flow rate of 5µL/min directly into the mass spectrometer. Mass spectra in full scan acquisition mode were acquired within the mass range of *m/z* 200-2000. All the detected MS *m/z* values possessing minimum normalized intensity of 10% were considered relevant. Between MS analysis, the injection system and MS equipment were washed with ACN in order to avoid possible carryover. MS/MS analysis of all the selected precursors was done by CID using helium as a collision gas, with a normalized collision energy of 30%, an activation time of 30ms and a radio-frequency of 0.250. ESI-tandem MS was controlled by Xcalibur v 2.0 software which was used also to perform data analysis.

Based on previous trials involving *A. rubens* CFF asterosaponins characterization, the *m/*z values searched in the obtained MS spectra were 1143, 1157, 1227, 1243, 1257, 1273, 1373, 1389 and 1405. Each asterosaponin was only considered present in the sample when the compound intensity were two-fold the background noise value. We estimated the percentage of background noise visually, multiplied that value by the higher absolute intensity in that spectrum and divided it by one hundred. We also considered that each asterosaponin was present in an experimental group when they were detected in at least two of the three replicates/pools.

- **Nano LC-MS/MS**

The CFF and RNC samples were analyzed on a Maxis Impact q-TOF spectrometer (Bruker, Bremen), coupled to a nano-HPLC system (Proxeon, Denmark). One µg of each injected sample dissolved in 5% ACN and 0.1% FA in water, were first concentrated on a 100 µm ID, 2cm Proxeon nanotrapping column and then loaded onto a 75 µm ID, 25 cm Acclaim PepMap nanoseparation column (Thermo). The chromatography was run using a 0.1% FA - ACN gradient (2-30% in 120 min for total lysates digests at a flow rate 300nL/min). The column was coupled to the mass spectrometer inlet through a Captive Spray (Bruker) ionization source. MS acquisition was set to cycles of MS (2Hz), followed by 3 second cycles of MS/MS (4-16Hz, intensity depending) of a variable number of the most intense precursor ions, with an intensity threshold for fragmentation of 2000 counts, and using a dynamic exclusion time of 2 min, with an automated precursor re-selection when a 3 fold increase in intensity was observed. All spectra were acquired on the range 150-2200 Da. LC-MS/MS data was analyzed using the Data Analysis 4.2 software (Bruker).

CFF proteins were quantified using MaxQuant 1.5.2.8 software. For RNC data, we did not had replicates and for that reason it was not possible to quantify. CFF and RNC proteins were identified using the Data Analysis 4.2 software (Bruker) to interpret LC-MS/MS and ProteinScape 3.1 using MASCOT search engine (version 2.2) and an echinoderm database generated by us using NCBI

protein sequences for all five classes. MS/MS spectra were searched with a precursor mass tolerance of 10 ppm, fragment tolerance of 0.05 Da, trypsin specificity with a maximum of 2 missed cleavages, cysteine carbamidomethylation set as fixed modification and methionine oxidation as variable modification. Significance threshold for the identifications was set to $p<0.05$, minimum MASCOT ions score of 20. After, samples were subjected to Blast2GO 3.1.2 for the assignment of information about the biological function and cellular location, by making use of NCBI BLAST web service, swissprot database and a blast expectation value of 0.001.

- **Statistical analysis**

The homogeneity and/or heterogeneity of number of leading arm movements of each sea star submitted to two pH conditions and 5 regeneration time-points were tested using Student's t-test with 95% confidence. The intensity values of proteins' cell-free coelomic fluid obtained from MaxQuant were used to check if they were present in each pH condition. If this was confirmed, a t-test (95% confidence) between the control pH and low pH was performed to verify if they were significantly different.

- **Behaviour trials**

The average number of leading movements of amputated and non-amputated sea star arms (AA and NAA, respectively) in two environmental conditions (control and acidic pH) and in 4 post-amputation time-points (A1, A4, A9 and A14) is discriminated in **figure 7**. Statistical tests comparing no-ablation (NA) and each post-ablation (PA) time-point for each arm group and pH condition are only presented in **Supplement 1**.

In the present study, *A. rubens* did not choose a preferential arm to lead the movement neither for CpH (p=0.93) or for LpH (p=0.23). As described by Ji et al. (2012) [40] and Cole (1913) [41], under free crawling conditions arm 5 seems to be the leading arm for *A. amurensis* and *A. forbesi*, respectively. Although, Ji et al. observed a more pronounced use of this arm in a fleeing situation that could be explained by a concentration of sense organs and nervous system in anterior side, supporting the theory of a bilateral behaviour in sea stars. Inconsistencies between our results and theirs may be due to different experimental procedures and criteria used for leading arm record. Regarding pH effect, Collard et al., (2013) [5] did not observed any influence in the mechanical properties of *A. rubens* tube feet.

In our study, one day after ablation the number of movements of AA were significantly higher at CpH, but without differences for the remaining time-points. No differences were observed between pH conditions for NAA at any time-point. When comparing both arm groups, NAA were responsible for a significant higher number of movements at both pH conditions, except for CpH/A9 and for

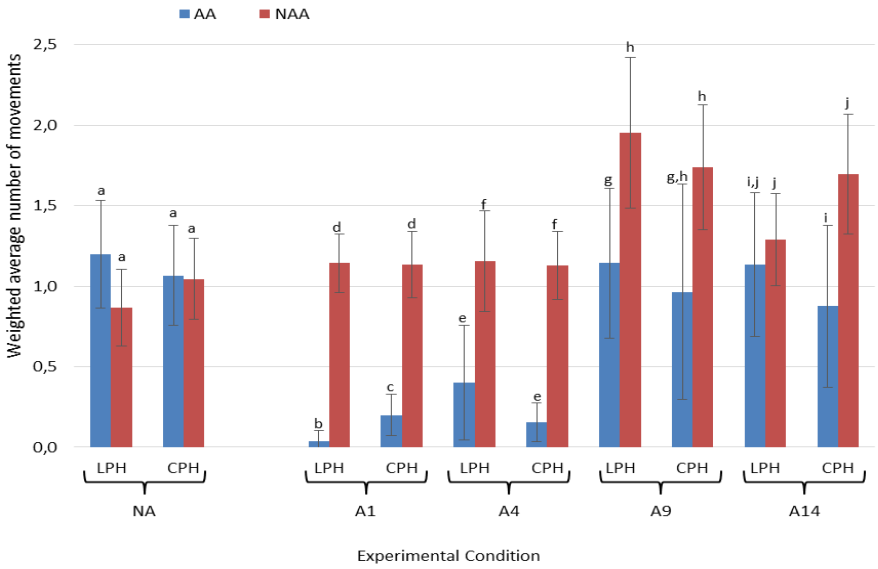

Figure 7. Weighted average number of movements of amputated and non-amputated arms (AA and NAA) in two pH experimental groups (CpH and LPH), and four PA time-points (A1, A4, A9 and A14). Different letters are used for statistically different observations in the same time-point only for intra and inter pH conditions, respectively between the two arm groups and within each arm group (see **Supplement 2**).

LpH/A14. Since day 9, AA increased their number of movements in both pH conditions, returning to the values observed for the NA condition. The NAA increased significantly the number of leading movements at this time-point and subsequently. The fact that we did not observed differences in NAA between pH conditions at any studied time-point is supported by several studies. Wood et al., (2011) showed that the artic brittlestar *Ophiocten sericeum* did not show difference in the percentage of functionally recovered arm regrown between the three pH treatments studied (7.3, 7.7 and 8.3) when subjected to an ambient temperature [42]. Although, for another brittlestar, *Aphiura filiformis,* an increase of arm regeneration rate was observed at an acidic pH, together with a decrease in muscular mass. Furthermore, Schram et al., (2011) reported no significant differences of arm regeneration rates between two pH treatments (7.8 and 8.2) for the asteroidea *Luidia clathrate*. The reduced seawater pH had no effect on the biochemical composition of the body wall of regenerating or intact arms, and it appears that despite the combined stress of regeneration and exposure to CO_2-driven seawater acidification, *L. clathrata* was able to consume sufficient nutrients to support functional regeneration of lost arms. It is known that lack of adequate nutrition resulted in preferential allocation of energy to the development of the pyloric caeca of intact arms, with subsequent energy secondarily allocated to arm growth [43]. In our work, sea stars were feed *ad libitum,* which mean they also did not had this constrain, and for that reason, they maintained the regeneration rate.

- **Analysis of CFF asterosaponins using UF-SPE-ESI-MS approach**

The main purpose of the experimental work using CFF's low molecular mass fraction (<5 kDa) was to detect and quantify the asterosaponins present in regenerating sea stars submitted to CpH and LpH conditions after 15 days in a regeneration situation.

Fractions 55% and 75% were injected and analyzed by ESI-MS. An example of a mass spectrum for each pH condition are presented in **Supplement 3**, and the fragmentation patterns MS^2 and MS^3 for *m/z* 1243 are shown in **Supplement 4** also as an example. A list of mass losses of all detected asterosaponins is presented in **Table 4**. Nine asterosaponins were detected in CpH, whereas only two of these were detected in LpH conditions (*m/z* 1243 and 1257; **Table 3**). Identifications were made based only on $[M-H]^-$ *m/z* values and their fragmentation patterns. Retention times could have been also used to have a more reliable identification, since the same *m/z* value can have different retention times [44], and consequently have a different molecular composition. In another sea star species submitted to regeneration, *M. glacialis,* asterosaponins with *m/z* 1227, 1259, 1243, 1373 and 1389 were present [14]. Only one more was detected when no amputation was inflicted (*m/z* 1405). This last asterosaponin was also detected in our samples from CpH/A15.

Table 3. Asterosaponins identification from sea stars submitted to different pH conditions (CpH and LpH) and 15 days post-amputation. Shaded cells correspond to asterosaponins detected in both environments. When two identifications are between parenthesis, they correspond to the same molecular structure, [a] [44], [b] [26], [c] [45].

m/z **$[M-H]^-$**	**Asterosaponin identification**
1143	Unidentified
1157	Unidentified
1227	Regularoside B [b]
1243	(Forbeside B / Glycoside B2), Thornasteroside A [b]
1257	(Forbeside C / Asterosaponin 1) [a], Asteroside C [a], Ruberoside F [a]
1273	Unidentified [a]
1373	Asteriidoside C [a]
1389	Unidentified
1405	(Forbeside A / Versicoside A) [a]

Table 4. Fragmentation patterns of all asterosaponins detected in CpH/A15.

m/z **$[M-H]^-$**	**MS^2**
1143	$[M-146]^-$
1157	$[M-18]^-$
1227	$[M-100]^-$
1243	$[M-100-146]^-$
1257	$[M-100-18]^-$ $[M-114]^-$
1273	$[M-100-18]^-$
1373	$[M-100]^-$
1389	$[M-100]^-$
1405	$[M-100]^-$

Quantification of asterosaponins detected in both pH conditions was performed using asterosaponin:peptide ratio (**Supplement 5**). It was observed that the ratios for *m/z* 1243 and 1257 decreased from mean values of 14 and 21 in CpH to values of 3 and 2 in LpH group, respectively (a decrease of around 4 and 9 times, respectively). Although there is a high variation coefficient value, a decrease was observed for all LpH replicates and for both asterosaponins.

All together these data show that a decline of 0.4 pH units cause a decrease on the number of detected asterosaponins. The two asterosaponins detected in both pHs, *m/z* 1243 and 1257, were also among the most abundant in a Demeyer et al. (2014) [44] report. They showed their presence in all *A. rubens* body components studied (aboral body wall, oral body wall, stomach, pyloric caeca and gonads). This is also true for *m/z* 1227 and 1273. Both studies revealed that asterosaponins concentrations differ greatly among individuals, which corresponds to a huge biological variability in this species. There is also known that sea star' asterosaponins present seasonal fluctuation, having a higher concentration during June and July [25]. Furthermore, Demeyer et al. (2014) [44] confirmed

significant diversity of asterosaponins among organs, being detected a larger number in oral and aboral body walls and a higher concentration in the latter and in the gonads. In the literature it is also suggested that in sea star, asterosaponins may play a role as a spawning inhibitor [46], an important feature for the control of reproduction.

Considering that *A. rubens* present a lower respiration rate after 27 days at pH 7.7 than at pH 8.1 [5], we can hypothesize that at time-point A15 the cellular respiration was diminished, leading to a lower ATP availability, which could compromise asterosaponins biosynthesis. These events may explain our results, in which a decrease of the number of asterosaponins detected was observed, but also a reduction in the concentration of the ones present in acidic pH environment. This may have negative impact on their digestive function, by reducing the ability to absorb cholesterol. Although, there are positive effects in their chemical signaling functions, enhancing predation ability when an 'escape response' event is involved.

- **Analysis of CFF proteins using Nano-LC approach**

Several works regarding *A. rubens* subjected to arm regeneration has been published (e.g.: ref [17]) but not at the proteomic level. One limitation when performing this type of approach is the absence of an annotated genome for Asteroidea. For this reason the current study is, in its majority, a homology-driven proteomic characterization, usually with *S. purpuratus,* the closest phylogenetic relative with a sequenced genome. In this study, 66% of all identified proteins are homologs to the proteins of this sea urchin. As previously mentioned, we performed relative quantification of CFF proteins using mass spectrometry data and MaxQuant software. Only two of the 31 proteins identified were differentially expressed between pH conditions ($p<0.05$), showing a significant intensity increase at low pH (**Table 4**). This event can correspond to a positive response from the organism, since these proteins, Vitellogenin 1 and alpha-tubulin1, are respectively responsible for deposition of yolk substances in the oocytes [47], and for cytoskeleton structure [48]. Actin was expressed in both pH conditions.

Since we reported a decrease in asterosaponins at an acidic pH, and knowing that these molecules are spawning inhibitors [46], the increase in vitellogenin expression is required to couple with an intensive oocytes production. Additionally, Dupont et al., (2010) showed for *C. papposus*, larvae and juveniles raised at low pH grow and develop faster. The combined effects of an increase in vitellogenin expression and a decrease in asterosaponins biosynthesis appears to contribute to an enhanced reproductive ability at an acidic pH.

As mentioned before [49], *A. rubens* present an increased cellular proliferation at a control pH environment between 7 and 21 days post-amputation. Consequently, this will involve cytoskeleton

biogenesis. Eventually, at an acidic pH for A15, cellular proliferation increases and a higher alpha-tubulin expression will be need for the cytoskeleton formation.

Table 4. Statistical analysis of proteins' quantification results: t-Student tests were performed in LFQ intensity values of proteins present in CpH and LpH environments. Significant differences were considered for p<0,05.

NCBI accession number	Description	CpH (relative intensity)	LpH (relative intensity)	p-value
587759606	vitellogenin 1 [*Patiriella regularis*]	910.000	8.529.200	0,011
21667221	alpha-tubulin 1, partial [*Strongylocentrotus droebachiensis*]	653463	2.555.500	0,018

After Blast2GO analysis, we compared GO terms annotations from all CFF proteins identified at both CpH and LpH samples, and no differences were observed among the several classes (**Figures 7 and 8**). Data show that the most represented **biological processes (BP)** are biological regulation, response to stimulus, cellular component organization or biogenesis, and cellular, developmental, metabolic, multicellular organismal and single-organism processes. The two major **molecular functions (MF)** represented are binding and catalytic activity. All these GO terms are represented by more than 6% and 10% of all identified proteins, for BP and MF, respectively. For more details on GO terms see **Supplement 6**.Although no differences were observed in the GO terms, differences are obvious when comparing the list of proteins (**Figure 9**). From 186 proteins identified in LpH group and 168 in CpH group, 56 are common (a detailed table for all proteins identified, "CFF protein identification", is supplied in CD-ROM). It is worth to mention that the list of proteins used to create the GO terms' graphs is not the same used to construct the Venn diagram. Shortly, B2G homology search was done against a general database without taxonomy restrictions, so we only used it for GO annotation. Although more than one hundred proteins are different between pH conditions (figure 9), this is not reflected in GO terms distribution (figures 7 and 8). The total number of CFF proteins identified in our study (298), is in agreement with Dheilly et al., (2013) [48] that identified 307 proteins in *S. purpuratus,* although trough different methods.In this work, as in ours, many proteins had multiple homologs, which included actin, tubulin and heat shock proteins. From 298 proteins identified, 227 were "Predicted", 54 "uncharacterized", 6 "putative" and 4 "hypothetical".

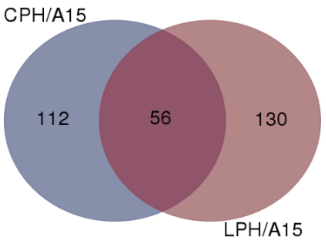

Figure 9. Number of identified CFF proteins detected in common and in each pH condition.

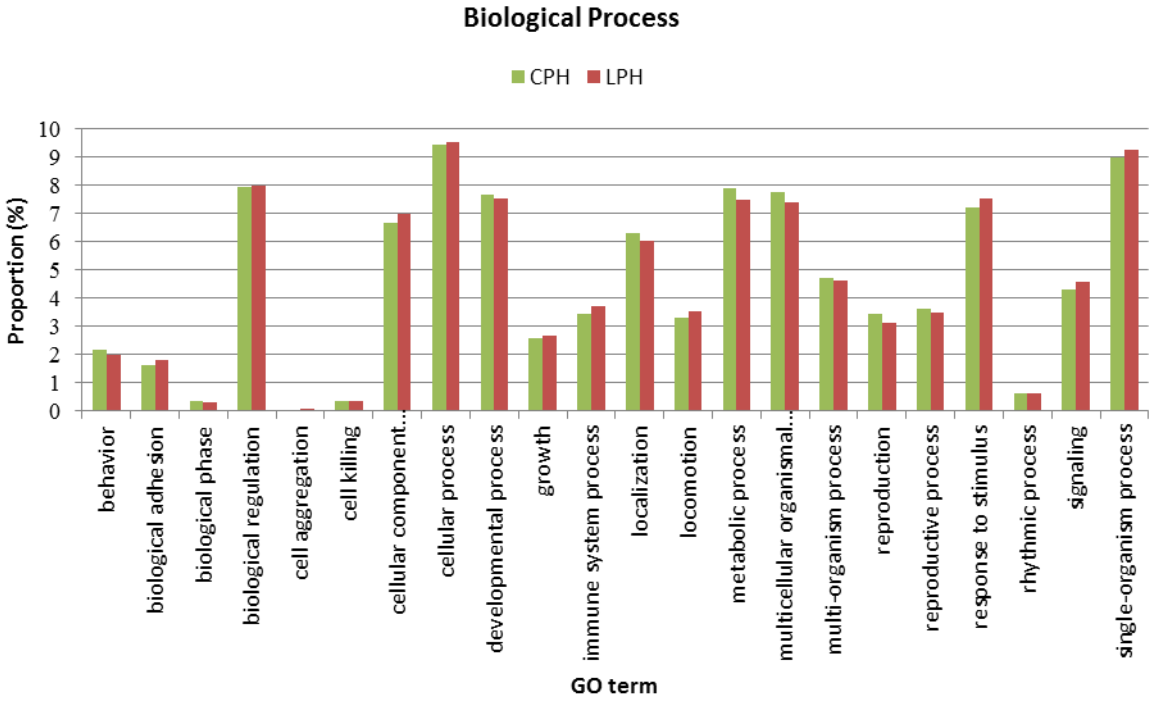

Figure 7. Gene Ontology (GO) biological process classification of all proteins identified from Blast2GO.

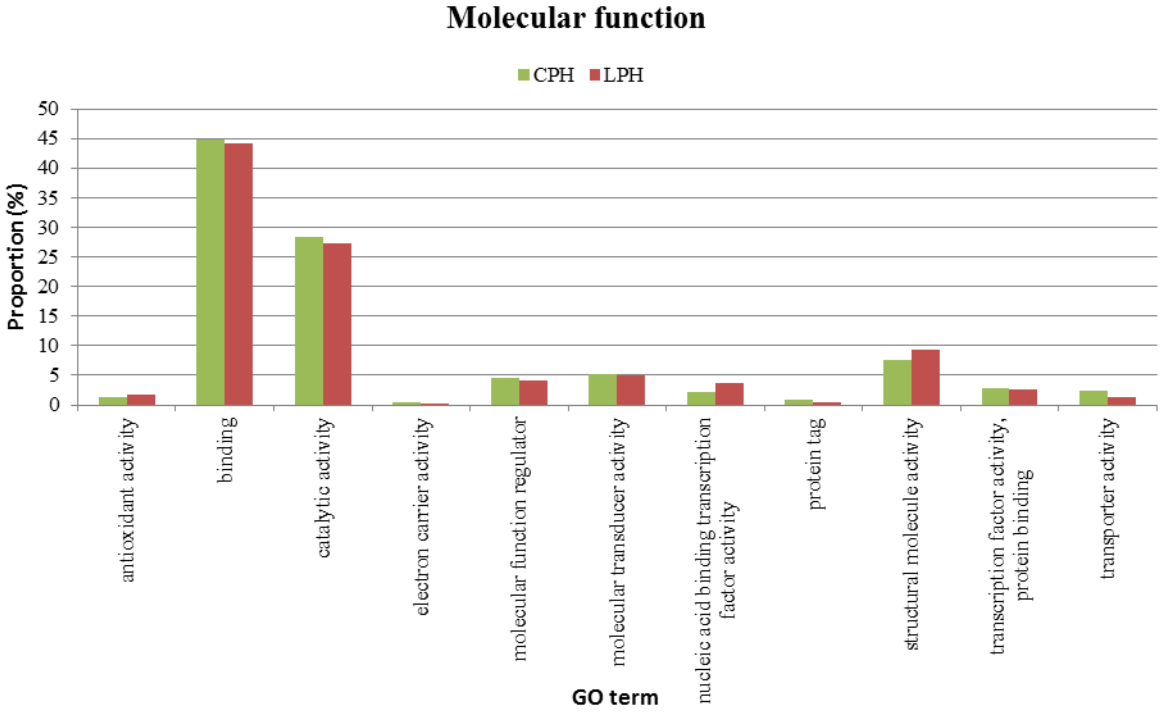

Figure 8. Gene Ontology (GO) molecular function classification of all proteins identified from Blast2GO.

As expected, it were detected **Heat Shock Proteins (HSP)**, or stress proteins. Although, we were predicting an HSP expression increase under a low pH condition, based on recent reports concerning corals and zooplankton [50, 51]. But surprisingly, they were found only at CpH (HSP90-alpha-isoform, HSP70 protein 1-like, heat shock cognate 71kDa protein or Hsc71), with exception of one that was present at the LpH condition (heat shock 70 kDa protein IV-like). General functions of stress proteins include protein folding, peptide chaperone, cytoprotection, intracellular signaling, cell-cycle control and buffering of harmful mutations [52]. Besides HSPs, nine **ubiquitin** homologs were found in both pH conditions, as expected. Attachment of a short chain of ubiquitins, for example, to a lysine, tags the protein for intracellular proteolytic destruction by a proteasome [53]. This is reported for *M. glacialis* for a regeneration condition [54].

Next, we will discuss the proteins identified in only one of the pH conditions studied, and in their majority also identified in Dheilly et al. (2013) [48] work, as seen in supplements provided in the CD-ROM.

- CFF proteins detected in control pH

In this condition it were identified **myosin V**, **interaptin, nesprin-1, dynein heavy chain 3 and 5,** and **avdillin** as being involved in the dynamic properties of actin filaments and cytoskeletal modifications. Involved in immune response we found **complement C2-like**, that is a part of the complement system, which evolved as a supplement of the innate immune system. This protein is, among others, responsible for the formation of an enzymatic activity called a C3 convertase, that eventually will help opsonize pathogens, and induce a series of inflammatory responses that help to fight infection [53]. A transport protein **SFT2**, responsible for vesicle-mediated transport was found, as for a **Na+/K+ ATPase alpha-1 subunit**, which is located in the membrane to act like a sodium/potassium-exchanging ATPase activity. We also found **sodium channel and clathrin linker 1**, **sodium/potassium/calcium exchanger 2 isoform X1** and **sodium/glucose cotransporter 4**. A very important protein involved in cell cycle control, **cyclin-dependent kinase 1** was also identified.

- CFF proteins detected in low pH

In pH 7.7 we found **Ras-related protein Rab-33 isoform X1** and **Ras-related protein Rab-10 isoform X1**, which are small GTPases responsible for regulating intracellular membrane trafficking and formation of transport vesicles to their fusion with membranes. Acting in cell signaling it were identified **hexokinase** and **low-density lipoprotein receptor-related protein 6**. In humans, **apoptosis-inducing factor 2**, a flavoprotein oxidoreductase, binds single stranded DNA and is thought to contribute to apoptosis in the presence of bacterial and viral DNA. The expression of the gene that encode this protein is also found to be induced by tumor suppressor protein p53 in colon cancer

cells. Involved in stress response, it was identified **catalase**, which help protect against the destructive effects of reactive oxygen species (ROS). Only identified in this pH was **lysozyme**, an enzyme abundant in most vertebrates body fluids, lyses both Gram-positive and negative bacteria, and interact directly or indirectly to modulate the complement activation cascade [48]. **Histone H3** and **histone H2A** are involved in cell cycle. The former plays a key role in guaranteeing tension-sensing function in mitosis, while the latter may be required for proper centromere function during chromosome segregation in *S. cerevisiae*. **Talin-1 isoform X2** are known to link integrins to the actin cytoskeleton, inhibits cadherin transcription and is involved in phagocytosis in mammals. But in echinoderms its function is not clear. Also acting in an actin-based cytoskeleton we identified **cingulin-like protein 1**. **Tenascin-R-like** is an extracellular matrix glycoprotein of the central nervous system. **Hamartin** is possibly a tumor suppressor. **Chymotrypsin B** is a pancreatic serine proteinase known to cleave substrates selectively at peptide bonds formed by some hydrophobic residues, like tryptophan, phenylalanine and tyrosine. **Beta-arrestin-1** is essentially signal transductor inhibitor.

To better understand the effect of a low pH environment in each protein identified here, more studies have to be perform detailing the biological impact of the differential expressions observed.

- **Analysis of Radial Nerve Cord proteins using Nano-LC approach**

For protein extraction/digestion of the radial nerve cord a new approach was used. The nano LC-MS/MS allowed identification of 213 proteins, although only one was common to all conditions studied (**Figure 10**). This result lead us to conclude that an optimization of the experimental procedure used should be performed.

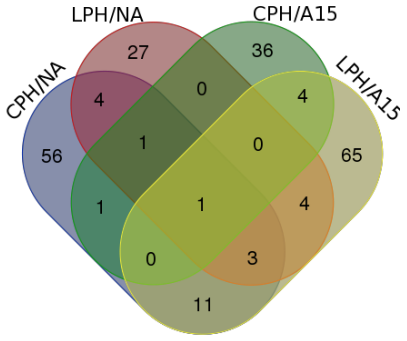

Figure 10. Number of identified RNC proteins detected in common and in each regeneration time-point and in each pH condition.

CONCLUSIONS

The aim of this work was to study the effect of the future predicted ocean acidification conditions under arm regeneration in the common sea star, *Asterias rubens*. First, it was observed their preference in choosing a leading arm when subjected to several regeneration time-points under a control and an acidic pH. It was concluded that they do not have a preferred leading arm before ablation in both pHs. Although, one day after amputation, and as expected, the number of movements of the amputated arms drastically decrease, being significantly higher the control pH condition. Since day 9, amputated arms restored their initial number of movements, and non-amputated arms increased it. We can conclude that at this time-point, normal movement was recovered.

We also searched for the presence of asterosaponins, molecules that have digestive, reproductive and chemical signaling functions. We observed that two of the nine molecules detected in the control pH, were also detected in low pH samples (*m/*z 1243 and 1257). Furthermore, their concentration decreased, suggesting that acidic pH may alter their biosynthesis. Nevertheless, further studies are needed to understand the effect of these lower levels of asterosaponins in *A. rubens'* biological processes.

Finally we performed a differential proteomic approach on the radial nerve cord and cell-free coelomic fluid tissues. The former analysis suggests an optimization of the experimental procedure used. Although, CFF analysis allowed identification of 298 proteins, of which 19% were common to both conditions. According to these results, 81% of all proteins identified were differentially expressed among pH 7.7 and 8.1, and future studies should emphasis this aspect in order to better understand their functions.

Merging all the results, we propose a correlation between an acidic environment and asterosaponins' biological effects. The decline in diversity and amount of these biomolecules can be caused by already known decreases in respiratory rate and cholesterol absorption. Although, asterosaponins might have positive effects in predation and reproduction. The detected increase in vitellogenin expression seems to be associated with the amplified spawning event induced by the low levels of asterosaponins at acidic pH, thereby also enhancing reproduction.

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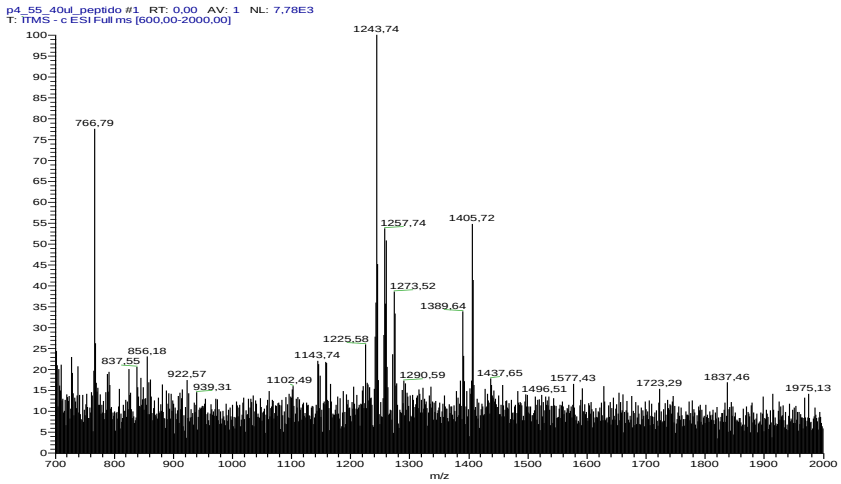
SUPPLEMENT 1: Statistical analysis of behaviour trial results: t-Student tests were performed between initial conditions (NA) and each PA time-point (A1, A4, A9 and A14) within each arm group (NAA & AA) and pH condition (CPH & LPH). Significant differences were considered for p<0,05 (shaded cells).

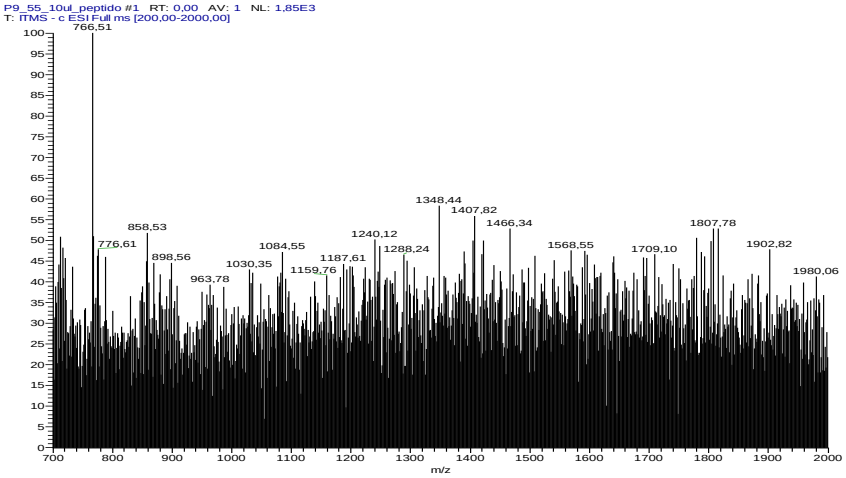
LPH		**CPH**		**LPH**		**CPH**		**LPH**		**CPH**		**LPH**		**CPH**	
AA & A1		**NA & A1**		**NA & A4**		**NA & A4**		**NA & A9**		**NA & A9**		**NA & A14**		**NA & A14**	
AA	NAA	AA	NAA	AA	NAA	AA	NAA	AA	NAA	AA	NAA	AA	NAA	AA	NAA
0,0000	0,1432	0,0002	0,6508	0,0098	0,2042	0,0003	0,6869	0,8648	0,0008	0,7996	0,0131	0,8370	0,0568	0,5753	0,0211

SUPPLEMENT 2: Statistical analysis of behaviour trial results: t-Student tests were performed for each time-point between pH conditions (CPH & LPH) for each arm group, and between arm groups (NAA & AA) for each pH condition. Significant differences were considered for p<0,05 (shaded cells).

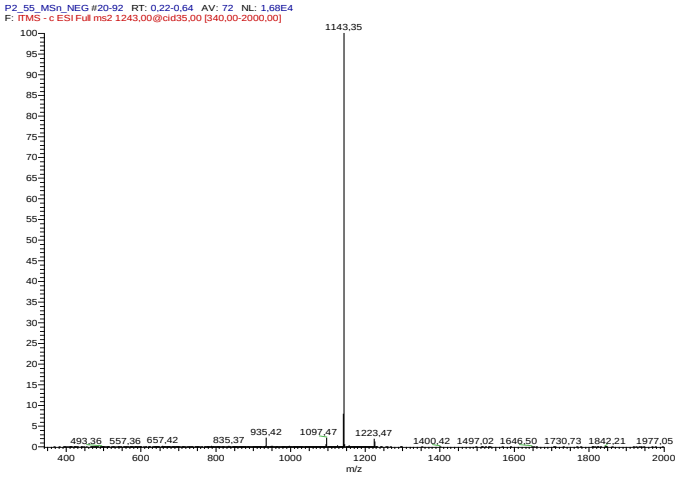
NA			**A1**			**A4**			**A9**			**A14**		
Common condition	Compared conditions	p-value	Common condition	Compared conditions	p-value	Common condition	Compared conditions	p-value	Common condition	Compared conditions	p-value	Common condition	Compared conditions	p-value
AA	LPH & CPH	0,66	AA	LPH & CPH	0,04	AA	LPH & CPH	0,25	AA	LPH & CPH	0,68	AA	LPH & CPH	0,49
NAA		0,43	NAA		0,95	NAA		0,90	NAA		0,51	NAA		0,12
LPH	NAA & AA	0,23	LPH	NAA & AA	0,00	LPH	NAA & AA	0,00	LPH	NAA & AA	0,03	LPH	NAA & AA	0,58
CPH		0,93	CPH		0,00	CPH		0,00	CPH		0,07	CPH		0,03

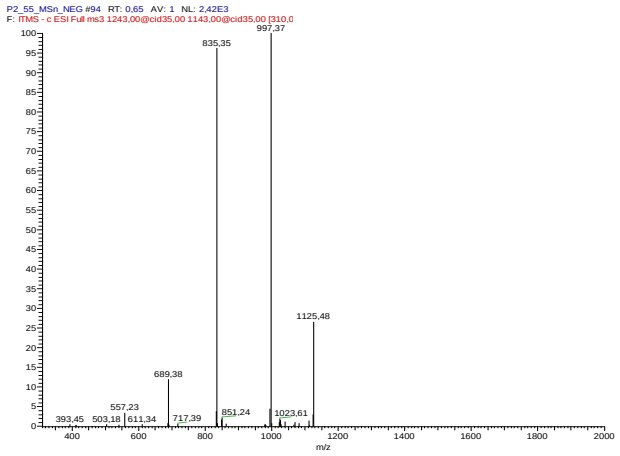
SUPPLEMENT 3: ESI-MS spectra of CFF extracted, fractions 55%. (left) CpH condition (Pool 4). (right) LpH condition (Pool 9).





SUPPLEMENT 4: (left) MS^2 fragmentation pattern of *m/z* 1243 showing a mass loss of 100 Da. (right) MS^3 fragmentation pattern of *m/z* 1243 showing a mass loss of [M-100-146-162]; [M-100-146] and [M-100-18] Da.





SUPPLEMENT 5: Asterosaponin:peptide ratio for three biological replicates of pH experimental conditions (CpH and LpH). Shaded cells correspond to asterosaponins detected in both pH conditions.

		Asterosaponins (*m/z*)								
	Pool	**1143**	**1157**	**1227**	**1243**	**1257**	**1273**	**1373**	**1389**	**1405**
Control pH	**3**	0,99	1,21	2,00	26,03	47,14	3,79	1,81	1,57	2,38
	4	0,50	0,47	0,69	7,84	6,88	1,19	0,64	0,80	1,29
	5	1,55	1,51	1,17	8,88	9,67	2,11	0,75	1,70	3,17
	Mean	1,01	1,06	1,29	14,25	21,23	2,36	1,07	1,36	2,28
	Standard Deviation	0,52	0,54	0,66	10,22	22,48	1,32	0,64	0,49	0,95
	Variation Coefficient	51,54	50,44	51,49	71,69	105,90	55,97	60,28	36,12	41,60
Low pH	**8**				3,68	1,81				
	9				0,79	0,75				
	11				5,08	4,61				
	Mean				3,19	2,39				
	Standard Deviation				2,19	1,99				
	Variation Coefficient				68,78	83,35				

SUPPLEMENT 6: Percentage of identified proteins for each GO term at both pH conditions (CpH and LpH).

	Biological Process Ontology	CpH	LpH
GO:0007610	behavior	2,17	1,95
GO:0022610	biological adhesion	1,60	1,78
GO:0044848	biological phase	0,33	0,27
GO:0065007	biological regulation	7,93	7,95
GO:0098743	cell aggregation	0,00	0,06
GO:0001906	cell killing	0,33	0,31
GO:0071840	cellular component organization or biogenesis	6,63	6,99
GO:0009987	cellular process	9,42	9,51
GO:0032502	developmental process	7,64	7,49
GO:0040007	growth	2,54	2,66
GO:0002376	immune system process	3,43	3,67
GO:0051179	localization	6,30	6,00
GO:0040011	locomotion	3,26	3,53
GO:0008152	metabolic process	7,88	7,46
GO:0032501	multicellular organismal process	7,75	7,39
GO:0051704	multi-organism process	4,70	4,61
GO:0000003	reproduction	3,44	3,12
GO:0022414	reproductive process	3,60	3,45
GO:0050896	response to stimulus	7,20	7,51
GO:0048511	rhythmic process	0,61	0,61
GO:0023052	signaling	4,28	4,54
GO:0044699	single-organism process	8,97	9,24

	Molecular Function Ontology	CpH	LpH
GO:0016209	antioxidant activity	1,32	1,83
GO:0005488	binding	44,87	44,21
GO:0003824	catalytic activity	28,38	27,32
GO:0009055	electron carrier activity	0,39	0,29
GO:0098772	molecular function regulator	4,63	4,09
GO:0060089	molecular transducer activity	5,12	5,07
GO:0001071	nucleic acid binding transcription factor activity	2,23	3,59
GO:0031386	protein tag	0,83	0,43
GO:0005198	structural molecule activity	7,66	9,30
GO:0000988	transcription factor activity, protein binding	2,75	2,49
GO:0005215	transporter activity	2,41	1,39